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Maternal Hepatic Effects of 1,2,4,5 Tetrachlorobenzene in the Rat

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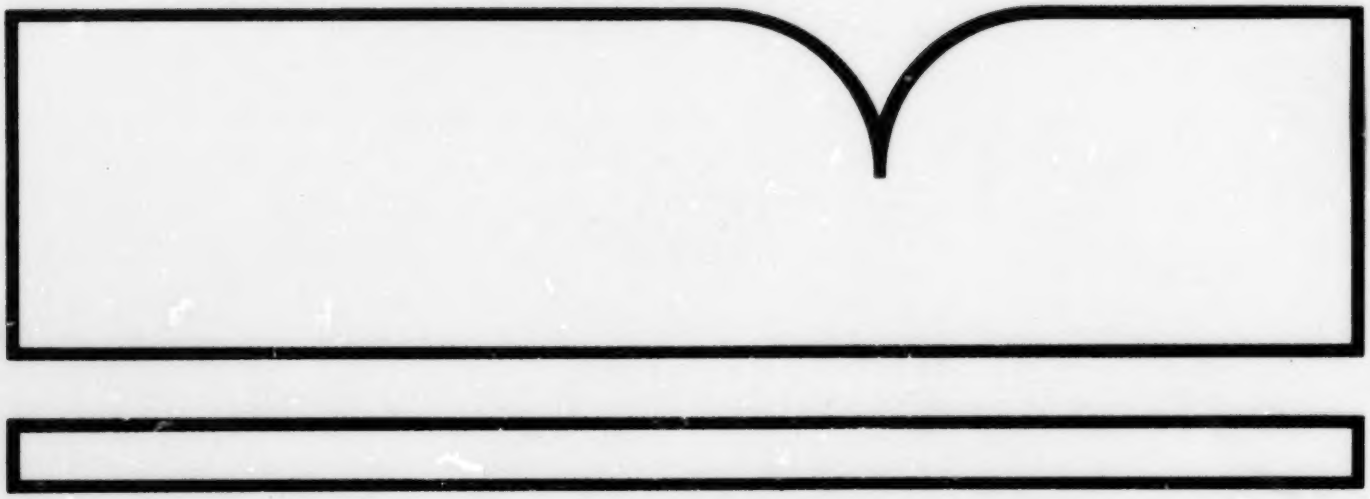

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16. ABSTRACT

1,2,4,5-Tetrachlorobenzene (TCB) is an industrial intermediate used in the production of 2,4,5-trichlorophenoxyacetic acid. This herbicide contains trace quantities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Because of possible maternal hepatic or reproductive effects of this uncharged, low-molecular weight, lipophilic chlorinated benzene 0, 30, 100, 300, and 1000 mg/kg/day of TCB was orally administered to rats on Days 9, 10, 11, 12, and 13 of gestation and the animals were sacrified on Day 14 of pregnancy. No maternal deaths were recorded and body weight gain was significantly decreased only in the 1000 mg/kg/day group. Maternal liver weight, liver to body weight ratio, and hepatic microsomal protein content were unaffected by TCB treatment. Although Day 14 NADPH-cytochrome c reductase activity was not affected, the maternal hepatic microsomal cytochrome *P*-450 content was significantly increased by administration of 1000 mg/kg/day of TCB. Microsomal *N*-demethylation of aminopyrine was slightly increased from 2.4 to 3.4 and 3.5 nmole/mg protein/min at doses of 300 and 1000 mg/kg TCB. However, maternal hepatic microsomal ethoxyresorufin *O*-deethylase activity was greatly increased from 14 to 30, 40, 50, and 49 pmole/mg protein/min in pregnant rats administered 0, 30, 100, 300, and 1000 mg/kg/day TCB. The microsomal rates of *p*-nitrophenol and phenolphthalein glucuronidation *in vitro* were not increased by TCB administration. The maternal hepatic microsomal enzyme induction observed after TCB administration to pregnant rats suggests the presence both cytochrome *P*-450 and *P*-448 inducers in the sample of 1,2,4,5-tetrachlorobenzene used.

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Maternal Hepatic Effects of 1,2,4,5-Tetrachlorobenzene in the Rat[1,2]

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1,2,4,5-Tetrachlorobenzene (TCB) is an industrial intermediate used in the production of 2,4,5-trichlorophenoxyacetic acid. This herbicide contains trace quantities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Because of possible maternal hepatic or reproductive effects of this uncharged, low-molecular weight, lipophilic chlorinated benzene 0, 30, 100, 300, and 1000 mg/kg/day of TCB was orally administered to rats on Days 9, 10, 11, 12, and 13 of gestation and the animals were sacrified on Day 14 of pregnancy. No maternal deaths were recorded and body weight gain was significantly decreased only in the 1000 mg/kg/day group. Maternal liver weight, liver to body weight ratio, and hepatic microsomal protein content were unaffected by TCB treatment. Although Day 14 NADPH–cytochrome c reductase activity was not affected, the maternal hepatic microsomal cytochrome *P*-450 content was significantly increased by administration of 1000 mg/kg/day of TCB. Microsomal *N*-demethylation of aminopyrine was slightly increased from 2.4 to 3.4 and 3.5 nmole/mg protein/min at doses of 300 and 1000 mg/kg TCB. However, maternal hepatic microsomal ethoxyresorufin *O*-deethylase activity was greatly increased from 14 to 30, 40, 50, and 49 pmole/mg protein/min in pregnant rats administered 0, 30, 100, 300, and 1000 mg/kg/day TCB. The microsomal rates of *p*-nitrophenol and phenolphthalein glucuronidation *in vitro* were not increased by TCB administration. The maternal hepatic microsomal enzyme induction observed after TCB administration to pregnant rats suggests the presence both cytochrome *P*-450 and *P*-448 inducers in the sample of 1,2,4,5-tetrachlorobenzene used.

INTRODUCTION

Chlorinated benzenes are industrial compounds present in the environment (Ware and West, 1977). The number and position of chlorine atoms on the benzene ring influences the lipid solubility, biotransformation, and toxicity of the molecule (Allen *et al.*, 1979; Ware and West, 1977; Ariyoshi *et al.*, 1975). The more highly chlorinated benzenes which lack the two adjacent hydrogen atoms required for rapid expoxidation (Kohli *et al.*, 1976) bioaccumulate in food chains. Hexachlorobenzene is an example of this unfortunate ecological property of highly chlorinated benzenes (Courtney, 1979). Chlorinated benzenes also possess low molecular weight and lack of charge which should permit substantial placental transport of these lipophilic compounds. Because chlorinated benzenes have been

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found in leaking chemical waste dumps, concern about their possible toxic, reproductive, or teratogenic effects has arisen.

1,2,4,5-Tetrachlorobenzene (TCB) is an industrial intermediate used in the production of the herbicide 2,4,5-trichlorophenoxyacetic acid. This herbicide is well known to contain variable quantities of the highly toxic (Harris *et al.*, 1973), teratogenic (Courtney and Moore, 1971), and carcinogenic (Kociba *et al.*, 1978; Pitot *et al.*, 1980) impurity 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD induces hepatic cytochrome *P*-448 and associated enzyme activities such as aryl hydrocarbon hydroxylase (Lucier *et al.*, 1973; Kitchin and Woods, 1978, 1979), biphenyl 2-hydroxylase (Hook *et al.*, 1975; Kitchin and Woods, 1979), and ethoxyresorufin *O*-deethylase (Kitchin and Woods, 1979) in female rats. Female rats are more sensitive to this type of enzyme induction than are males (Gurtoo and Parker, 1977; Lucier *et al.*, 1973), and quantities of TCDD as low as 0.002 μg/kg have elevated female rat hepatic aryl hydrocarbon hydroxylase activity (Kitchin and Woods, 1979). TCDD or 3-methylcholanthrene also induces conjugative drug metabolizing enzymes such as UDP-glucuronyltransferase (Lucier *et al.*, 1973, 1975; Wishart, 1978) activity toward *p*-nitrophenol.

Formation of reactive intermediates has recently been directly implicated as a mechanism of teratogenesis by investigations in an *in vitro* coupled microsomal activating/embryo culture system (Kitchin *et al.*, 1981). The overall concentration of metabolic reactive intermediates in various organs should be related both to the rate of formation (e.g., monooxygenase activities) in toxification reactions as well as the rate of detoxification or conjugation reactions (e.g., glucuronidation, sulfation, gluthatione conjugation) (Gillette, 1977). Some *in vivo* teratogenesis might be caused by hepatic formation of long-lived intermediates (e.g., 4-hydroxycyclophosphamide) (Connors *et al.*, 1974) and transport of these intermediates to the developing conceptus. Alternatively, reactive intermediates might be formed in embryonic or fetal tissues even though the rates of formation in these tissues are substantially less than that of the maternal animal (Hart *et al.*, 1962; Kitchin, 1980). Using 7½-day mouse embryos, Galloway *et al.* (1980) observed increases in the sister chromatid exchange frequency in benzo[α]pyrene-treated embryos. This effect was interpreted to be due to a low rate of benzo[α]pyrene metabolism in the embryonic tissue. Filler and Lew (1981) cultured preimplantation mouse embryos and detected benzo[α]pyrene metabolism *in vitro* and also demonstrated embryonic enzyme induction by TCDD in Day 3½ blastocysts.

In this study the maternal hepatic effects of administration of 30, 100, 300, and 1000 mg/kg/day of 1,2,4,5-tetrachlorobenzene were determined. TCB was administered on Days 9 to 13 of pregnancy and experimental animals were sacrificed on the 14th day. Day 14 embryos exposed to TCB *in vivo* were evaluated for growth and morphological parameters. The gestational chemical exposure period used in these studies is part of the period of susceptibility to teratogenesis in the rat (New, 1978). Present *in vitro* postimplantation embryo culture methods are also limited to this Day 9–14 period of gestation (New, 1978).

The results of this study and those of similar investigation with 1,2,3,4-tetra-

chlorobenzene and 1,2,4-trichlorobenzene can be used to test the hypothesis that chemically reactive intermediates may cause embryonic toxicity or teratogenicity. Unlike both 1,2,3,4-tetrachlorobenzene and 1,2,4 trichlorobenzene, 1,2,4,5-tetrachlorobenzene does not possess two vicinal hydrogens and should not form reactive epoxides.

METHODS

Experimental animals and dosing. Timed pregnant Sprague-Dawley rats (CD strain) were obtained from Charles River Breeding Laboratories (Kingston, N.Y.) and given food and water *ad libitum*. The day sperm were detected in the vaginal smear is referred to as Day 1 of gestation. 1,2,4,5-Tetrachlorobenzene (>98% pure; Aldrich Milwaukee, Wisc.) was ground to a fine powder with a mortar and pestle and suspended in 1.5% gum tragacanth (Sigma Chemical Co. St. Louis, Mo.). The suspensions were orally administered to pregnant rats on Days 9 to 13 of gestation in a volume of 2 ml/kg. Animals were killed by decapitation on the morning of Day 14 and their livers excised and weighed. Hematoxylin and eosin-stained sections of control and treated livers were examined histologically by standard procedures. In some studies the uteri were removed and the embryos examined for growth and differentiation parameters (Kitchin *et al.*, 1981). Live embryos were observed with a Wild 7A steromicroscope and scored as normal or abnormal as compared to gum tragacanth treated controls. The presence of beating heart, the somite number, and the embryo size were scored. After homogenization in 50 mM potassium phosphate buffer, pH 7.4, the total embryonic protein content was determined (Lowry, 1951) using bovine serum albumin (fraction V) as a standard.

Enzyme assays. Five grams of maternal rat liver was homogenized in 20 ml of 0.02 M Tris–HCl buffer (pH 7.5) containing 1.15 M KCl. Washed microsomes were prepared by ultracentrifugation as described by Kitchin and Woods (1978). Cytochrome *P*-450 content was assayed by the method of Omura and Sato (1964) using an Aminco DW-2 spectrophotometer. NADPH–cytochrome *c* reductase (LaDu *et al.*, 1971), aminopyrine *N*-demethylase (Nash, 1953; Lucier *et al.*, 1971), and ethoxyresorufin *O*-deethylase (Burke and Mayer, 1974) activities were determined by published procedures. The *in vitro* rates of the glucuronidation of *p*-nitrophenol (0.8 mM substrate) and phenolphthalein (0.2 mM substrate) were determined by substrate disappearance using 0.2% Triton X-100 activated microsomes (Mulder, 1970). The cofactor UDP-glucuronic acid was used at a concentration of 1.4 mM. The microsomal protein content was determined by biuret method (Gornall *et al.*, 1949) with bovine serum albumin (fraction V) as a standard.

Statistics. Analysis of variance procedures were used to determine statistically significant effects (Goldstein, 1964). A William's test (Williams, 1971) was used to determine dose-related effects while enumerative data was analyzed by the Fisher exact test (Goldstein, 1964). Quantitative data is presented as the mean ± SEM for five or more independent observations.

RESULTS

No maternal deaths were observed in this study. The body weight gain (Day 14 minus Day 8) of pregnant rats fed 1000 mg/kg TCB was significantly decreased from 34 to 15 g (Fig. 1). No significant treatment-related effects were observed on matenal liver weight, liver to body weight ratio, and hepatic microsomal protein content. Liver histology was normal in the control, 100, and 300 mg/kg TCB groups. In three of the nine female rats fed 1000 mg/kg/day TCB slight centrolobular hypetrophy was observed.

Maternal hepatic cytochrome *P*-450 content was significantly increased from 0.51 to 0.84 nmol/mg protein by administration of 1000 mg/kg/day TCB during Days 9–13 of pregnancy (Fig. 2). No increase in the activity of the flavoprotein NADPH–cytochrome *c* reductase was observed in this study. Two substrates were used to assay the *in vitro* activity of the hepatic monooxygenase system. The *N*-demethylation of aminopyrine (cytochrome *P*-450-type substrate) was increased from 2.35 to 3.43 and 3.53 nmole formaldehyde/mg protein/min by oral administration of 300 and 1000 mg/kg of TCB (Fig. 3). The maternal hepatic

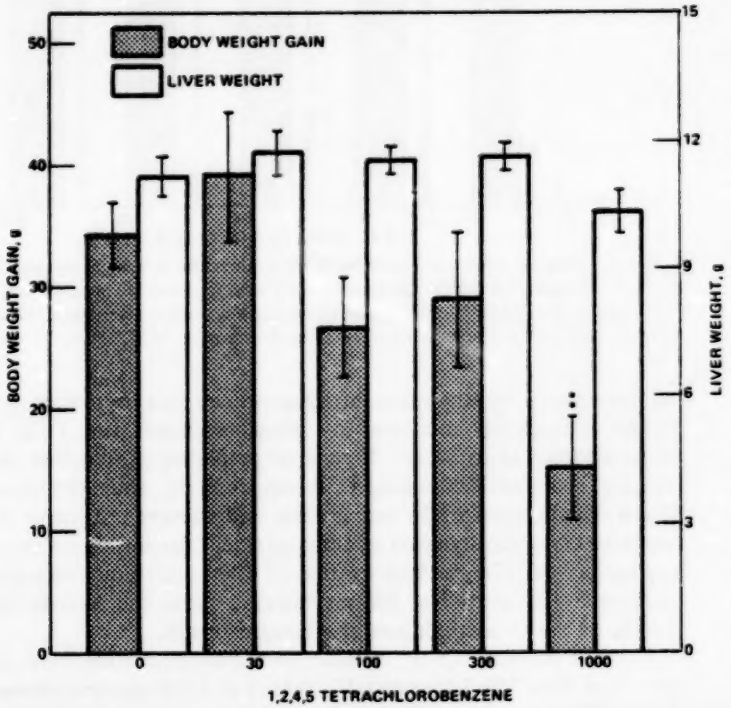

FIG. 1. Pregnant female rats were fed the indicated dose (mg/kg/day) of 1,2,4,5-tetrachlorobenzene during Days 9–13 of gestation. The body weight gain (gestational Day 14 minus Day 8) and Day 14 liver weights are expressed as the mean ± SEM for 6–8 animals. ** = $P < 0.01$.

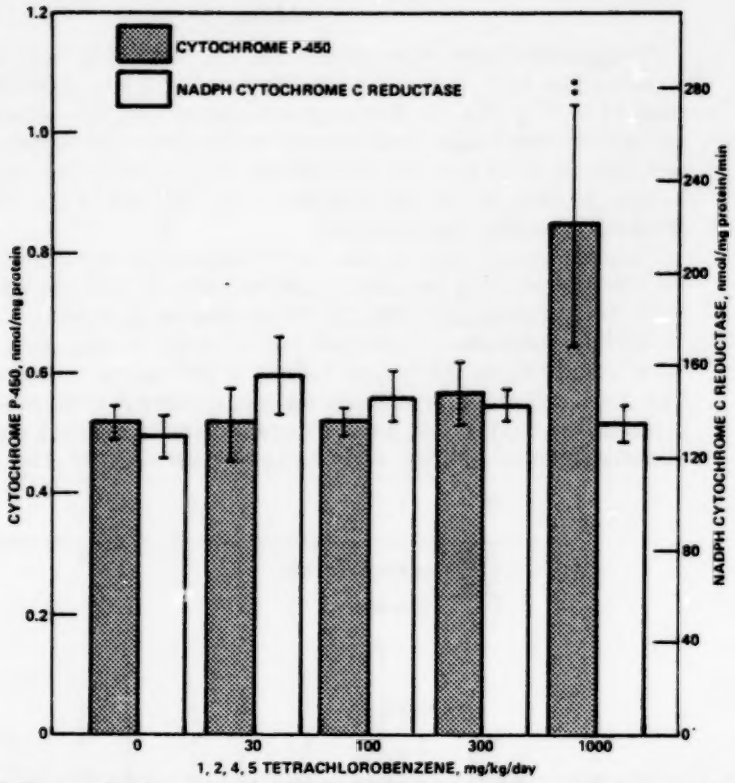

FIG. 2. Pregnant female rats were fed the indicated dose of 1,2,4,5-tetrachlorobenzene during Days 9–13 of gestation. On Day 14 the animals were sacrificed and the hepatic microsomal cytochrome *P*-450 content and NADPH–cytochrome *c* reductase activities determined. Six to eight animals were used in each group. The data is presented as the mean ± SEM. ** = $P < 0.01$.

microsomal *O*-deethylation of ethoxyresourifin (cytochrome *P*-448-type substrate) was greatly increased by prior treatment with TCB. The activity was elevated from 14 to 30, 40, 50, and 49 pmole/mg protein/min in the groups given 30, 100, 300, and 1000 mg/kg TCB, respectively. Thus, the increase in *O*-deethylation of ethoxyresorufin was greater and occurred at lower doses than did the increased *N*-demethylation of aminopyrine. Comparing the doses of 300 and 1000 mg/kg/day of TCB, administration of 1000 mg/kg/day increased hepatic cytochrome *P*-450 levels but did not increase either the *N*-demethylation of aminopyrine or the *O*-deethylation of ethoxyresorufin.

The two substrates *p*-nitrophenol and phenolphthalein were chosen to represent the "late fetal" and "neonatal" classes of UDP-glucuronyltransferases, respectively (Wishart *et al.*, 1978). After TCB administration to pregnant rats, the *in vitro* microsomal rates of glucoronidation toward *p*-nitrophenol and phenolphthalein were not altered.

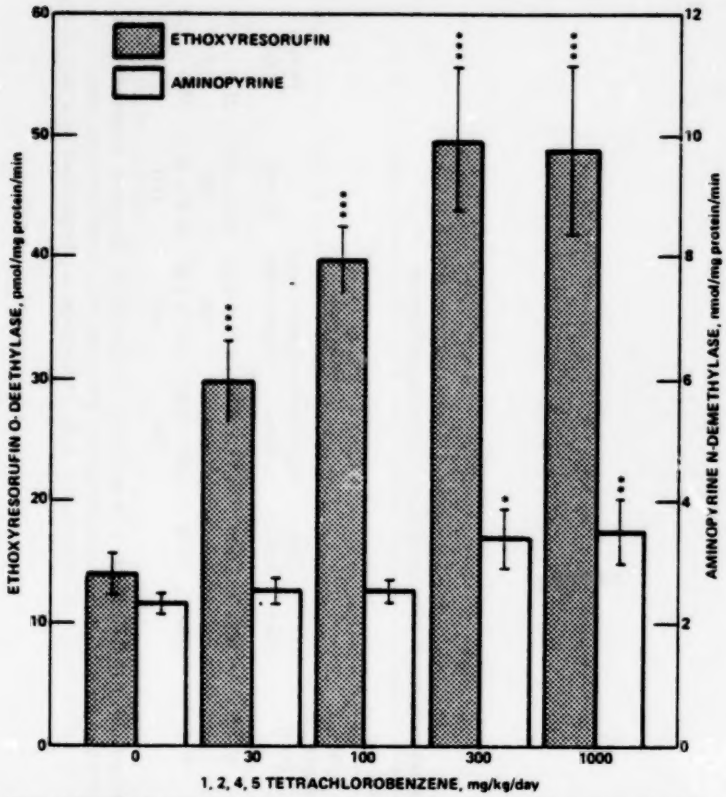

FIG. 3. Day 14 hepatic microsomal aminopyrine *N*-demethylase and ethoxyresorufin *O*-deethylase activities were determined after administration of several different doses of 1,2,4,5-tetrachlorobenzene to rats on Days 9–13 of gestation. Data is the mean ± SEM for 6–8 animals. * = $P < 0.05$, ** = $P < .01$; *** = $P < 0.001$.

Administration of 1000 mg/kg/day of TCB to pregnant rats during Days 9–13 of gestation did not adversely affect any Day 14 embryonic parameter examined. Resorptions, embryonic deaths, abnormalities, protein, somite number, crown to rump, and head length were not significantly altered by maternal TCB administration during organogenesis (Table 1). Neither yolk sac diameter nor protein content were affected by TCB treatment.

DISCUSSION

Overall TCB was not a highly toxic compound. At oral doses up to 1000 mg/kg/day TCB for 5 days no lethality occurred and a significant reduction in body weight gain was found only at the 1000 mg/kg/day dose. As 1,2,4,5-tetrachlorobenzene lacks two vicinal hydrogens it is expected that this compound would bioaccumulate in food chains and pose a greater health hazard from chronic rather

TABLE 1
EMBRYONIC GROWTH AND MORPHOGENESIS IN RATS GIVEN 1,2,4,5-TETRACHLOROBENZENE

		Resorptions		Embryonic					
	Implantations	Resorptions/ implantations	Affected litters/Total litters	Deaths	Abnor-malities	Head length (mm)	Crown–rump length (mm)	Somites	Protein (μg)
Vehicle	14.2 ± 0.3 (12)	9/161	5/12	0/26	0/26	4.72 ± 0.06 (26)	8.17 ± 0.09 (26)	49.6 ± 0.43 (26)	2170 ± 195 (26)
1,2,4,5-Tetra-chlorobenzene	12.3 ± 0.7* (12)	11/136	7/12	0/24	0/24	4.76 ± 0.08 (24)	8.33 ± 0.12 (24)	50.1 ± 0.40 (24)	2500 ± 220 (24)

Note. Dams were given daily oral doses of 1.5% gum tragacanth or 1000 mg/kg/day TCB on Days 9–13 of gestation. They were sacrificed by ether administration on the morning of Day 14 and the uteri examined for implantations and resorptions. Embryonic parameters were determined in two embryos from each maternal animal. With the exception of a lower number of implantations in the TCB group, no comparisons were statistically significant.

* $P < 0.05$.

than acute exposures. The related compound hexachlorobenzene is more toxic to suckling pups than it is to embryos *in utero* (Kitchin *et al.*, 1982; Grant *et al.*, 1977). Rat embryos during the organogensis phase of development were not adversely affected by TCB treatment. In similiar studies maternal administration of 1,2,3,4-tetrachlorobenzene (Kitchin and Ebron, 1983b) or 1,2,4-trichlorobenzene (Kitchin and Ebron, 1983a) during organogenesis caused embroyonic growth retardation. Thus, chemically reactive intermediates can be a cause of embryonic growth retardation.

The maternal hepatic microsomal effects of TCB were of a mixture of phenobarbital and 3-methylcholanthrene types of enzyme induction. The maternal hepatic increase in cytochrome *P*-450 content (65%) was less than the maximum possible in pregnant female rats (3.1-fold increase, Kitchin and Ebron, 1983b). Ethoxyresorufin *O*-deethylase rates were induced (up to 3.5-fold) but not to maximally inducible levels obtained with β-naphthoflavone administration (13-fold, Kitchin and Ebron, 1983b). This cytochrome *P*-448-linked (Burke and Mayer, 1975) enzyme activity was increased 2-fold, even at does as low as 30 mg/kg/day TCB. The cytochrome *P*-450 type of induction of TCB was shown by the increase in aminopyrine *N*-demethylation rate from 2.4 to 3.4 and 3.5 nmole/mg protein/min, respectively, at the two highest TCB doses.

Nonpregnant Wistar rats fed 250 mg/kg/day TCB for 3 days had increased hepatic microsomal protein levels, cytochrome *P*-450 content, and aminopyrine *N*-demethylase activities (Ariyoshi *et al.*, 1975). The differences between the Ariyoshi *et al.* (1975) study and the results presented here may be due to the strain of rat used and the pregnant, endocrine state of our animals.

It is surprising that TCB treatment did not result in an increase in UDP-glucuronyltransferase activity toward *p*-nitrophenol. In male and nonpregnant female rats *p*-nitrophenol glucuronidation is increased by 3-methylcholanthrene or TCDD administration (Wishart *et al.*, 1978; Lucier *et al.*, 1975).

Oxidative (ethoxyresorufin *O*-deethylase, aminopyrine *N*-demethylase) reaction rates were increased by TCB administration while conjugative enzyme rates (glucuronidation; glutathione *S*-transferase, data not presented) were unaffected. Administration of TCB did not reduce hepatic glutathione levels (Kitchin, unpublished data). This selective increase in toxification reaction rates could mean that the steady state hepatic level of reactive intermediates (e.g., from endogenous chemicals or exogenous molecules such as TCB) would be higher in a TCB-induced animal than in an unexposed control.

As both cytochrome *P*-450- and *P*-448-mediated reactions were induced by TCB, this suggests the presence of chemical inducers for both these isoenzymes. In general, hepatic microsomal-inducing chemicals fall into either the phenobarbital class or the 3-methylcholanthrene class and do not induce both sets of enzyme activities. The commercial sample of TCB used in these studies was >98% pure. Sophisticated analytical chemical analyses are required to identify and quantitate the minor and trace components of this TCB sample which could account for its dualistic enzyme induction properties. This pattern of enzyme induction occurs with mixtures of polychlorinated biphenyls and with some "pure"

TABLE 2
COMPARISON OF THE BIOLOGICAL EFFECTS OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN AND 1,2,4,5-TETRACHLOROBENZENE

	Result expected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin[a]	Result observed with 1,2,4,5-tetrachlorobenzene
Hepatic ethoxyresorufin *O*-deethylase	Induction	Induction
Hepatic UDP glucuronyl transferase activity for *p*-nitrophenol	Induction	No effect
Embryonic toxicity/ teratogenicity	Yes	No

[a] The results expected from TCDD administration are predicted from relevant published literature (Courtney and Moore, 1971; Kitchin and Woods, 1979; Lucier *et al.*, 1975).

compounds. Goldstein *et al.* (1978) fed four different doses of pure and technical hexachlorobenzene to female rats and determined the activity of several hepatic microsomal enzymes. Exposure to pure and technical hexachlorobenzene diets induced hepatic microsomal cytochrome *P*-450, aryl hydrocarbon hydroxylase, aminopyrine *N*-demethylase, and *p*-nitrophenol glucuronidation to the same extent at each dose level.

If toxicologically significant amounts of TCDD were present in the TCB sample used, three biological responses should have occurred (Table 2). TCDD should have induced hepatic ethoxyresorufin *O*-deethylase, increased UDP-glucuronyltransferase activity toward *p*-nitrophenol, and caused embryonic toxicity and teratogencity. As only hepatic microsomal ethoxyresorufin *O*-deethylase induction occurred and the other two biological responses did not, it is unlikely that TCB contains biologically significant levels of TCDD or other extremely toxic chlorinated aromatics.

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